

Supplementary Information

Capzimin is a potent and specific inhibitor of proteasome isopeptidase Rpn11

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Supplementary Results

Supplementary Table 1. Small molecule screening data

Category	Parameter	Description
Assay	Type of assay	In vitro biochemical assay
	Target	Rpn11 (NCBI Protein ID: PSMD14; Gene ID: 10213)
	Primary measurement	Fluorescent Polarization
	Key reagents	Ub4-pepOG protein substrate, 26S proteasome from human erythrocytes
	Assay protocol	See PubChem Bioassay: AID 588493 (https://pubchem.ncbi.nlm.nih.gov/bioassay/588493) In summary: uHTS Procedure 1) Using LabCyte Echo, transfer 40 nL of test compounds from a 2 mM compound source plate into assay plate Cols. 5-48 (final concentration of test compounds is 20 uM, 1 % DMSO). Transfer 40 nL of 100% DMSO into assay plate Col. 1-4. 2) Pre-incubate 20nM 26S proteasome in Epoxomicin Solution at room temperature for 1 hour, then dilute 10-fold in pre-chilled 1x Assay Buffer. 3) Using Beckman BiorapTR dispense 2uL of Assay Buffer in columns 1 and 2, and 2uL of 26S proteasome solution to columns 3-48. 4) Using Beckman BiorapTR dispense 2ul of Ub4-pepOG substrate into all wells (columns 1-48) 5) Spin plates at 1500 rpm for 1 minute on Eppendorf centrifuge 5810. 6) Incubate for 80 minutes at room temp. 7) Read plates on Perkin Elmer Envision with dual mirror at Ex/Em 480/535 nm in fluorescent polarization mode
Library	Library size	359.521
	Source	Molecular Libraries Small Molecules Repository Library (MLPCN, NIH)
Screen	Format	1,536 Well Plate Format
	Concentration(s) tested	20 μ M
	Reagent/ compound dispensing system	Labcyte Echo, Beckman Bioraptr
	Detection instrument and software	PerkinElmer Envision
	Assay validation/QC	Z'=0.63, signal to background = 1.6 fold, signal to noise = 12.5 fold, 5.9 signal window
	Correction factors	None
	Normalization	None
Post-HTS analysis	Hit criteria	≥ 40 % of inhibitory activity compared to the DMSO controls
	Hit rate	0.55%
	Additional assay(s)	(1) Against an out-of-family thrombin metalloprotease for selectivity and possible interference with the fluorescence polarization readout. (AID: 602333). (2) against MMP2 metalloprotease to exclude nonselective and potential likely Zn ²⁺ chelators (AID: 602330).

Supplementary Table 2. 8TQ and Capzimin are uncompetitive inhibitors of Rpn11 (proteasome holoenzyme)

Michaelis-Menten parameters				
8TQ	10 μ M	5 μ M	2.5 μ M	0
Vmax \pm S.E.	25.84 \pm 3.37	72.84 \pm 4.5	114.4 \pm 4.02	140.1 \pm 1.8
Km \pm S.E.	0.36 \pm 0.16	0.49 \pm 0.09	0.77 \pm 0.07	0.82 \pm 0.03
CZM	3.3 μ M	1.7 μ M	0.3 μ M	0
Vmax \pm S.E.	63.81 \pm 4.05	106.3 \pm 1.19	139.5 \pm 3.37	172.7 \pm 3.29
Km \pm S.E.	0.41 \pm 0.12	0.66 \pm 0.03	0.80 \pm 0.07	1.03 \pm 0.07

Supplementary Table 3. Summary of Capzimin’s IC50 on metalloenzymes

IC50 (μM) of Capzimin on other metalloenzymes				
HDAC6	MMP 2	MMP 12	hCAII	GLO1
>200	>50	>50	>50	42.8±2.2

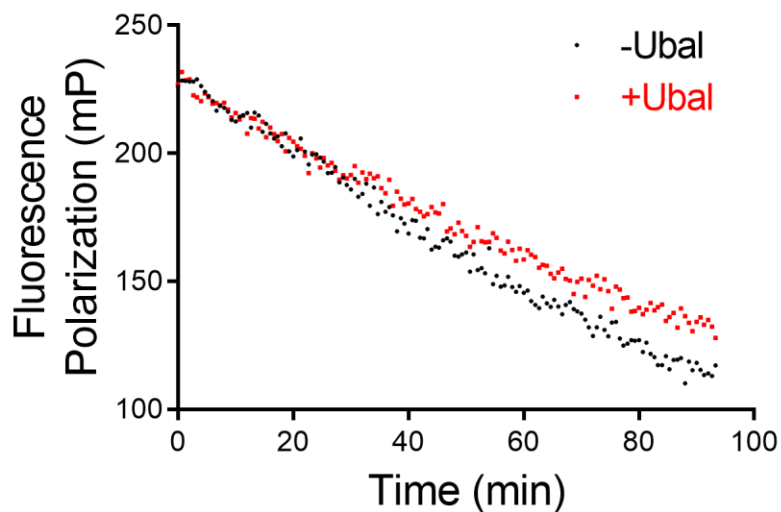
Supplementary Table 4. A subset of 3021-resistant clones have elevated proteasome activity and increased resistance to carfilzomib.

	3021 IC ₅₀ (μM)	CFZ IC ₅₀ (nM)	Peptidase activity (LLVY-AMC)
HCT116	2.2	7.1	100%
Line 4	5.3	60	144%
Line 5	2.7	11	127%
Line 6	6.2	45	130%
Line 9	4.2	16	153%

Supplementary Table 5. Antibodies used in this study

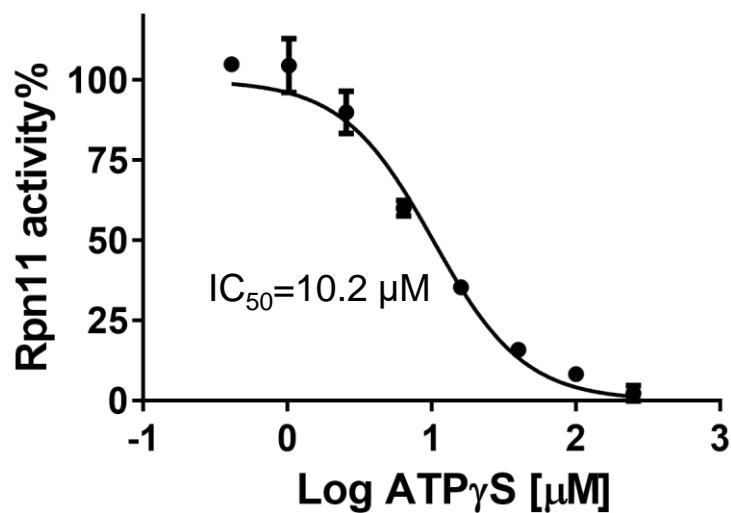
Antibody	Vendor and catalog No.	Applications
Ubiquitin pAb	Enzo, ADI-SPA-200-F	WB
Nrf2	Santa Cruz, sc-365949	WB
Cul1	Life Technologies, 32-2400	WB
Actin	Santa Cruz, sc-1616	WB
Ubiquitin	Santa Cruz, Sc-8017	IF
HDAC6	Cell Signaling, 7558S	IF
SQSTM1	Santa Cruz, sc-25575	IF
HIF1	BD bioscience, 610959	WB
p53	Cell Signaling, 2524S	WB
PERK	Cell Signaling, 5683P	WB
BiP	Cell Signaling, 3177P	WB
XBP1s	BioLegend, 647502	WB
CHOP	Cell Signaling, 2895P	WB
DNAJB4	Santa Cruz, sc-100711	WB
GAPDH	Santa Cruz, sc-25778 HRP	WB

Supplementary Figure 1



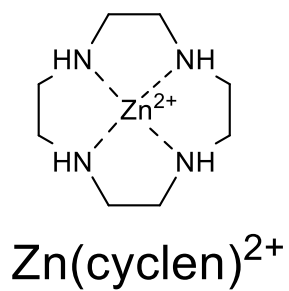
Supplementary Figure 1: Ub aldehyde has little effect on the kinetics of Ub₄peptide^{OG} cleavage. Shown are kinetic curves from the Rpn11 assay performed in the absence (black) or presence (red) of 1 μ M Ub aldehyde.

Supplementary Figure 2



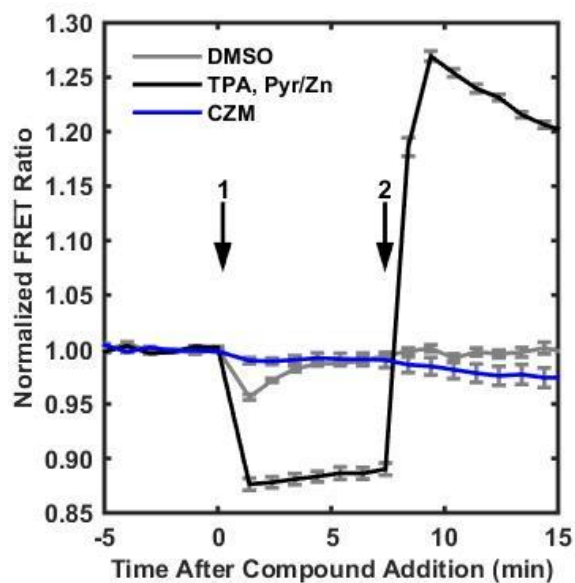
Supplementary Figure 2: Cleavage of Ub₄peptide^{OG} requires ATP hydrolysis. The IC_{50} for inhibition of Rpn11 activity by non-hydrolyzable ATP γ S was determined. Error bars represent s.d., $n=4$

Supplementary Figure 3



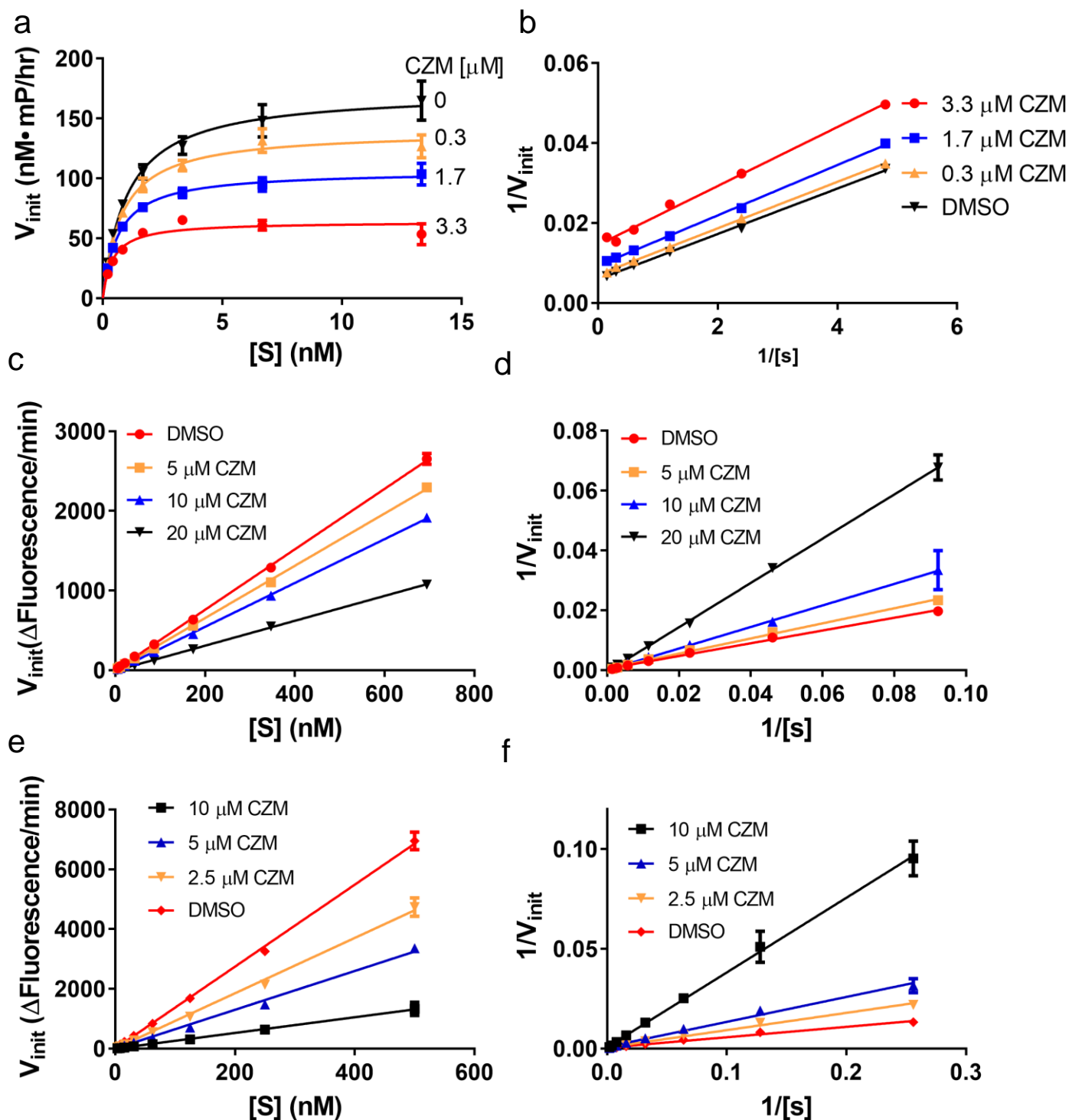
Supplementary Figure 3: Structure of Zn(cyclen)^{2+}

Supplementary Figure 4



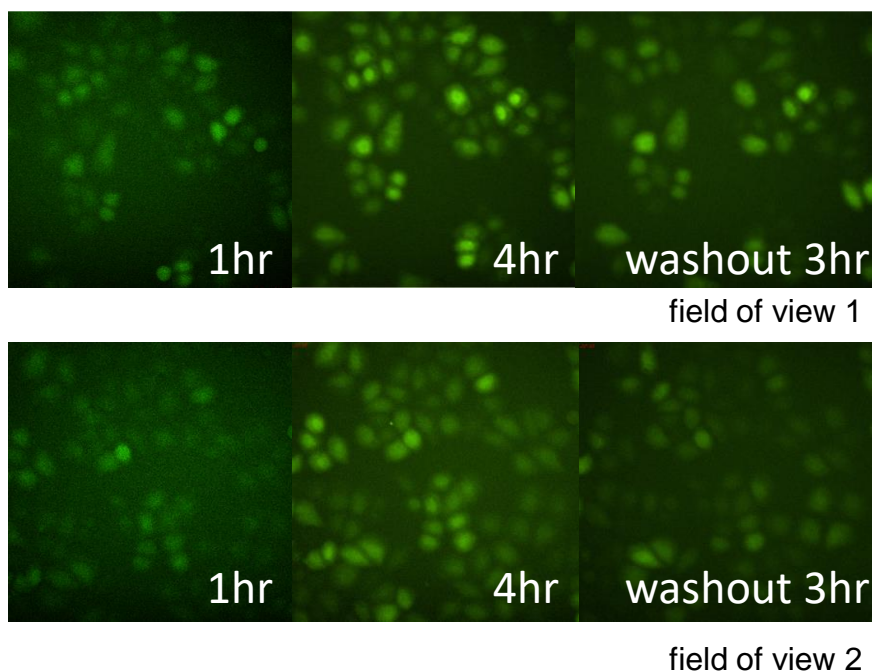
Supplementary Figure 4: Capzimin does not alter cellular zinc. Cells were treated with 0.5% DMSO (gray, $n=11$ cells), 50 μM TPA (black, $n=12$ cells), or CZM 10 μM (blue, $n=11$ cells) at arrow 1. At arrow 2, TPA-treated cells were washed and treated with 0.75 μM pyridine and 12 μM buffered zinc solution (Pyr/Zn).

Supplementary Figure 5



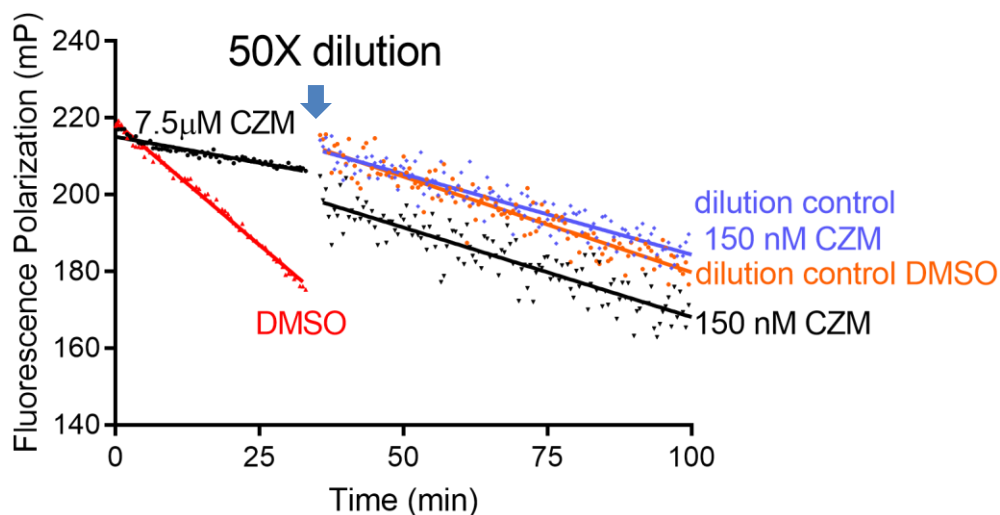
Supplementary Figure 5: CZM is an uncompetitive inhibitor of Rpn11 (proteasome holoenzyme), but a competitive inhibitor of AMSH and BRCC36. Shown are the Michaelis-Menten curves (a, c, e) or Lineweaver-Burke plots (b, d, f) of Rpn11 (a, b), AMSH (c, d) or BRCC36 (e, f) activity assay in the presence of different concentrations of CZM. Error bars represent s.d., n=3.

Supplementary Figure 6



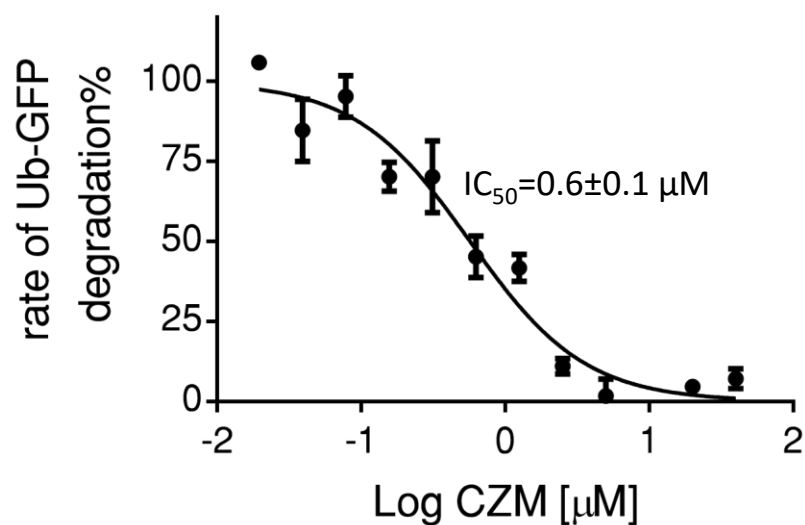
Supplementary Figure 6: Capzimin reversibly inhibits the proteasome in cells. Shown are fluorescence images of cells taken at different time points (as indicated) after treatment with 4 μ M of **CZM** or washout of **CZM**.

Supplementary Figure 7



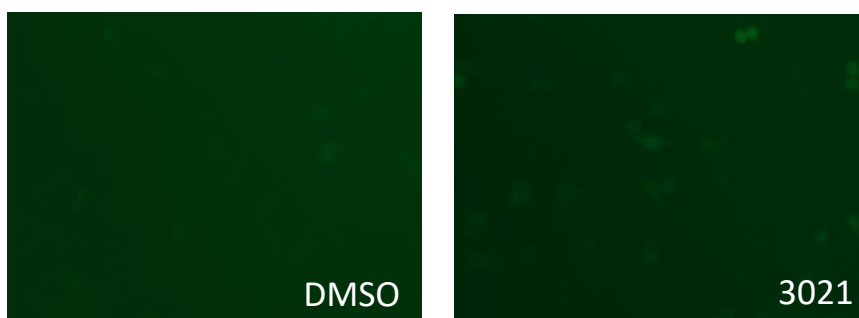
Supplementary Figure 7: Capzimin is a reversible inhibitor in vitro. Shown is the fluorescence polarization kinetics before and after 50X dilution. Before dilution, high concentration of CZM (black) showed >80% inhibition of Rpn11 activity compared to DMSO control (red). After 50X dilution, CZM (black) showed <10% inhibition of Rpn11 activity compared to the DMSO control (orange). A pre-diluted sample (blue) with same concentration of proteasome and CZM served as dilution control.

Supplementary Figure 8



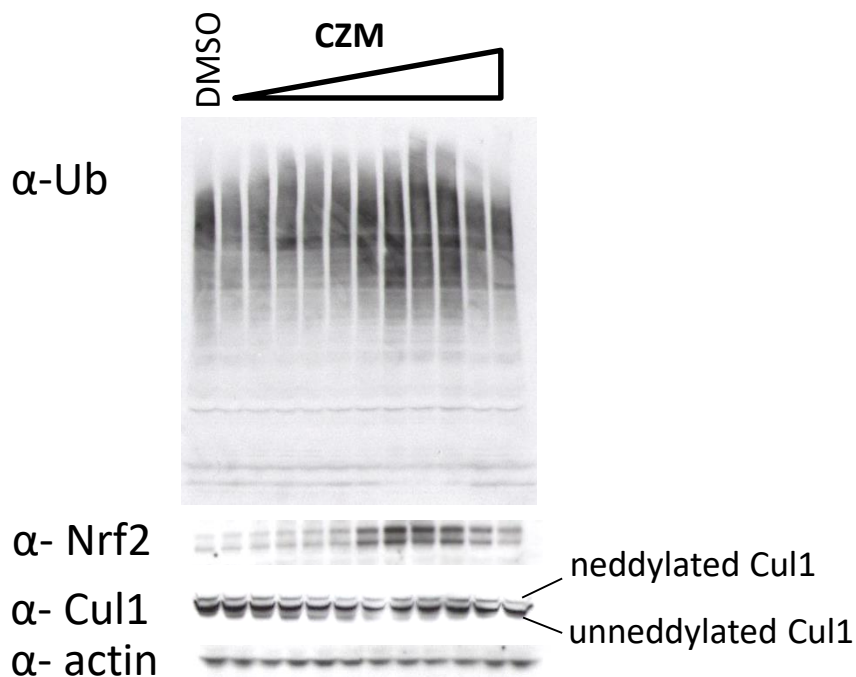
Supplementary Figure 8. Capzimin block the clearance of accumulated Ub^{G76V}GFP. The degradation rate of Ub^{G76V}-GFP was measured at different concentrations of capzimin by cycloheximide chase assay. Error bars represent s.d., n=3

Supplementary Figure 9



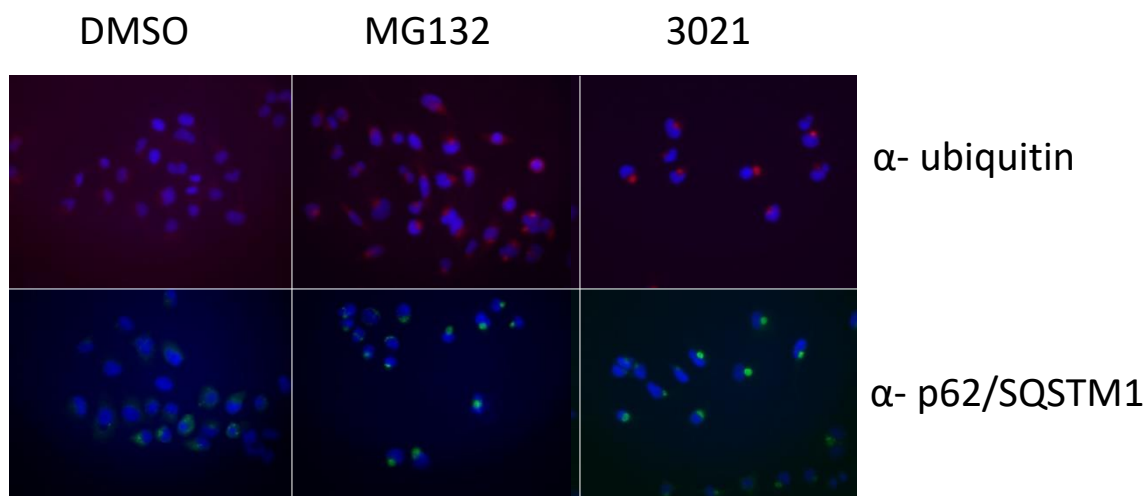
Supplementary Figure 9. 3021 has little effect on Ub^{G76V}GFP accumulation. Shown is a fluorescence image of cells taken 4 hours after treatment with DMSO or 5 μ M of **3021**.

Supplementary Figure 10



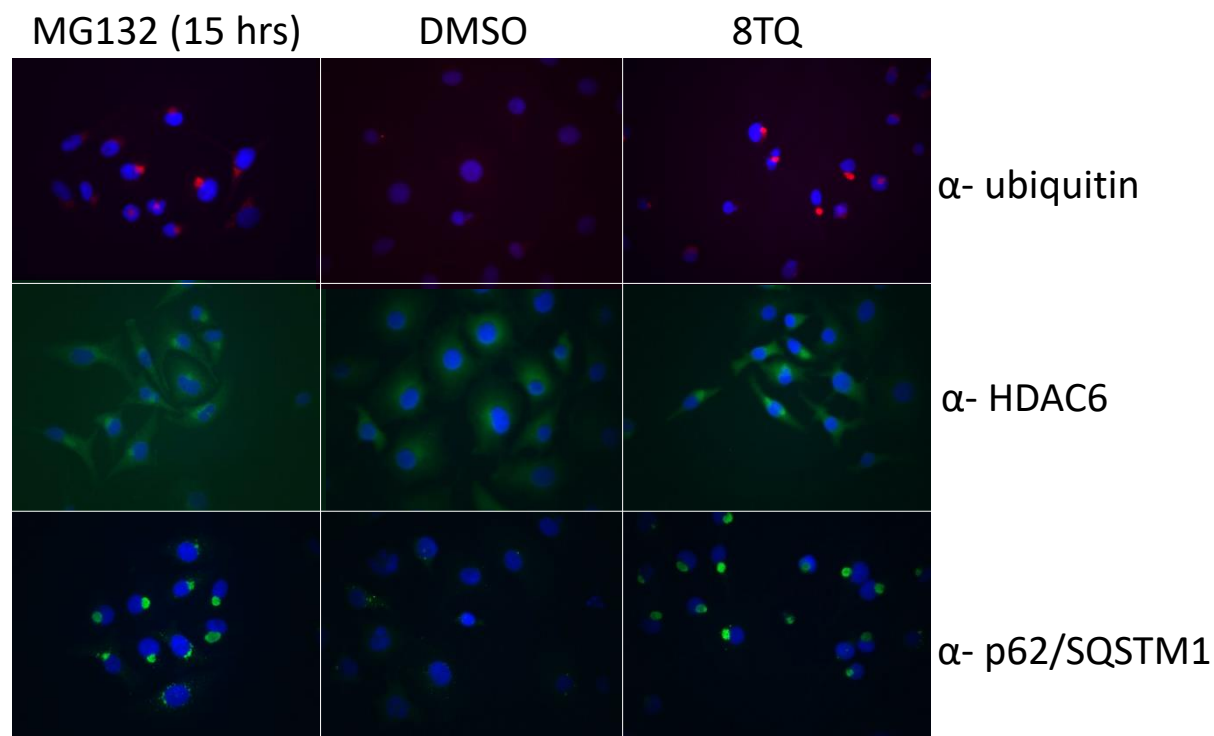
Supplementary Figure 10: Capzimin treatment induces accumulation of polyubiquitinated species and Nrf2 in 293T cells. 293T cells were treated for 2 hours with different concentrations (10 nM to 100 μ M) of capzimin and cell lysates were fractionated by SDS-PAGE and immunoblotted with antibodies against ubiquitin, Nrf2, Cul1 and actin, as indicated.

Supplementary Figure 11



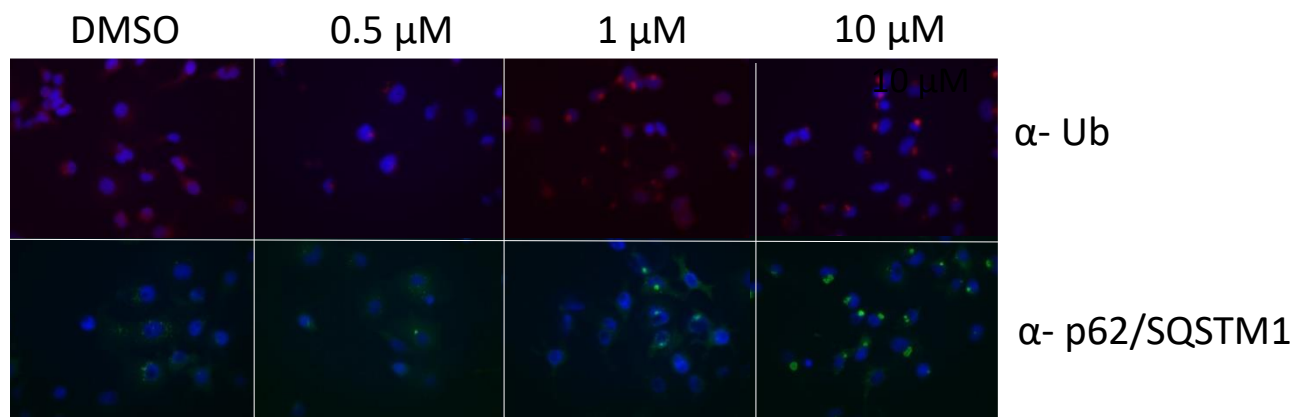
Supplementary Figure 11: 3021 induces aggresome formation. A549 cells were treated with DMSO, 5 μ M MG132 or 10 μ M **3021** for 15 hours before being fixed and stained with antibodies against ubiquitin or p62/SQSTM1.

Supplementary Figure 12



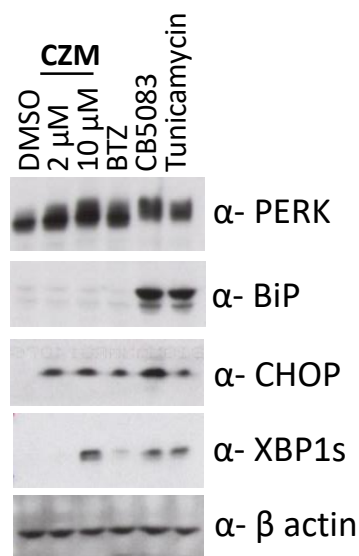
Supplementary Figure 12: 8TQ prevents the clearance of aggresomes. A549 cells were treated with 5 μ M MG132 for 15 hours to induce aggresome formation. MG132 was then washed away. The cells were treated with DMSO or 5 μ M 8TQ for 24 hours before being fixed and stained with antibodies against ubiquitin, HDAC6, or SQSTM1.

Supplementary Figure 13



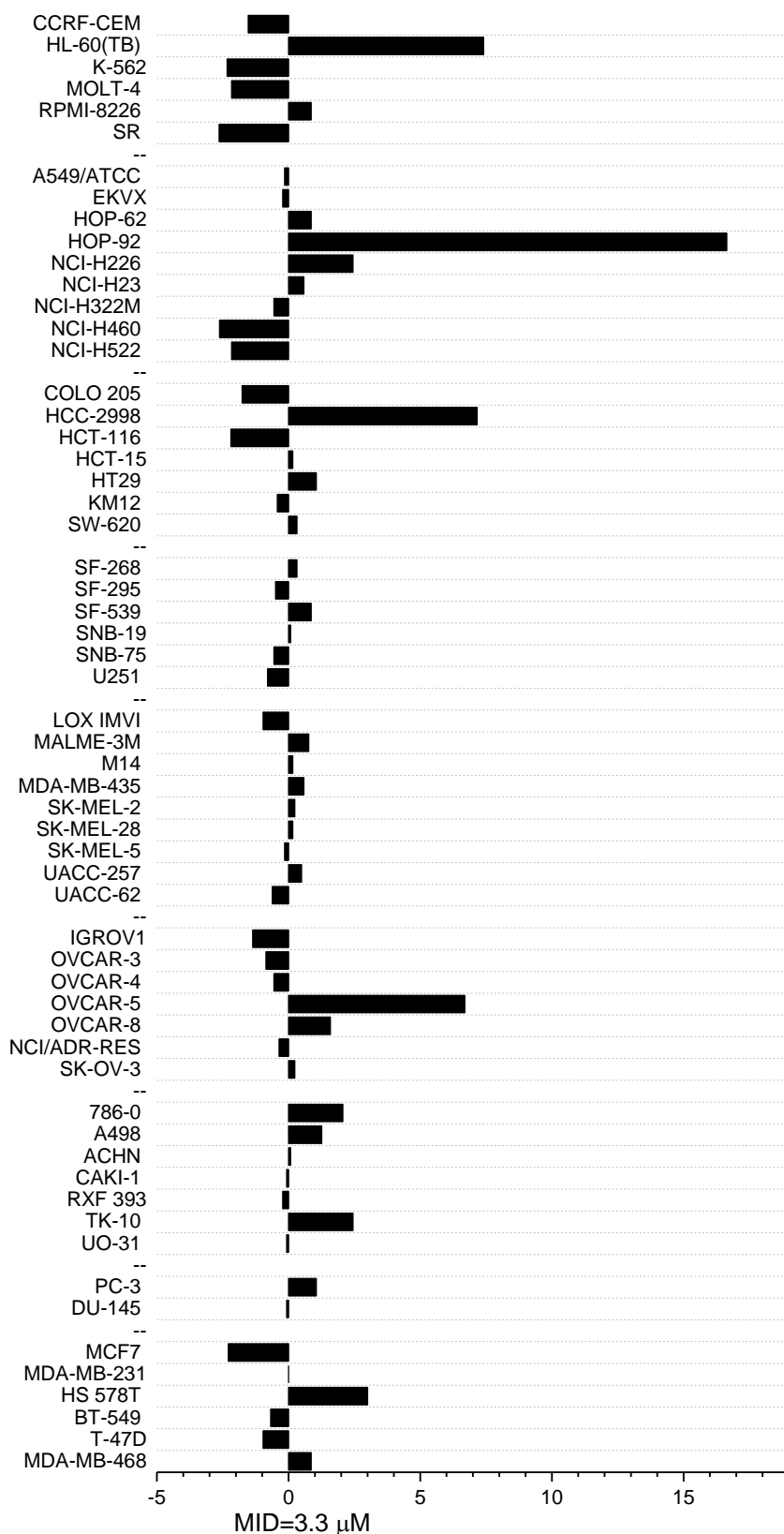
Supplementary Figure 13: 3021 prevents the clearance of aggresomes. A549 cells were treated with 5 μM MG132 for 15 hours to induce aggresome formation. MG132 was then washed away and cells were treated with DMSO or different concentration of **3021** for 24 hours before being fixed and stained with antibodies against ubiquitin or SQSTM1.

Supplementary Figure 14



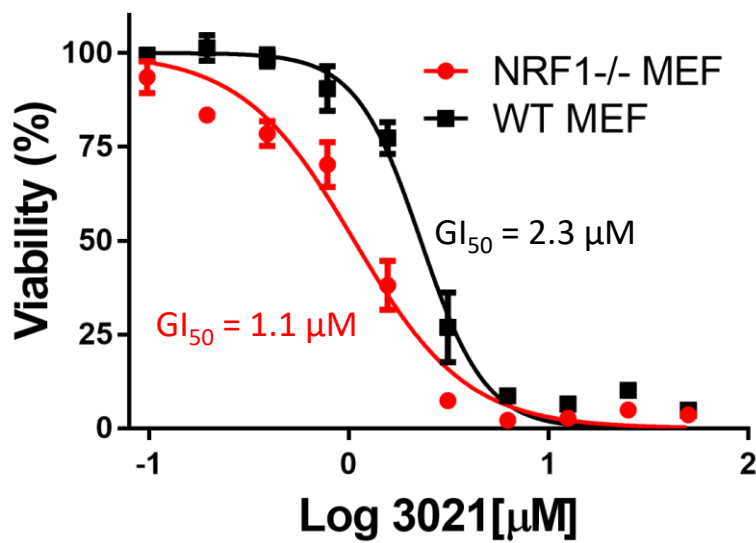
Supplementary Figure 14: Capzimin induces an unfolded protein response in 293T cells. 293T cells were treated for 8 hours with different concentrations of Capzimin (2 or 10 μ M), BTZ (1 μ M), the p97/VCP inhibitor CB5083 (10 μ M), or Tunicamycin (2 μ g/ml) and cell lysates were fractionated by SDS-PAGE and immunoblotted with antibodies against PERK, Bip, Xbp1s, CHOP and actin, as indicated.

Supplementary Figure 15



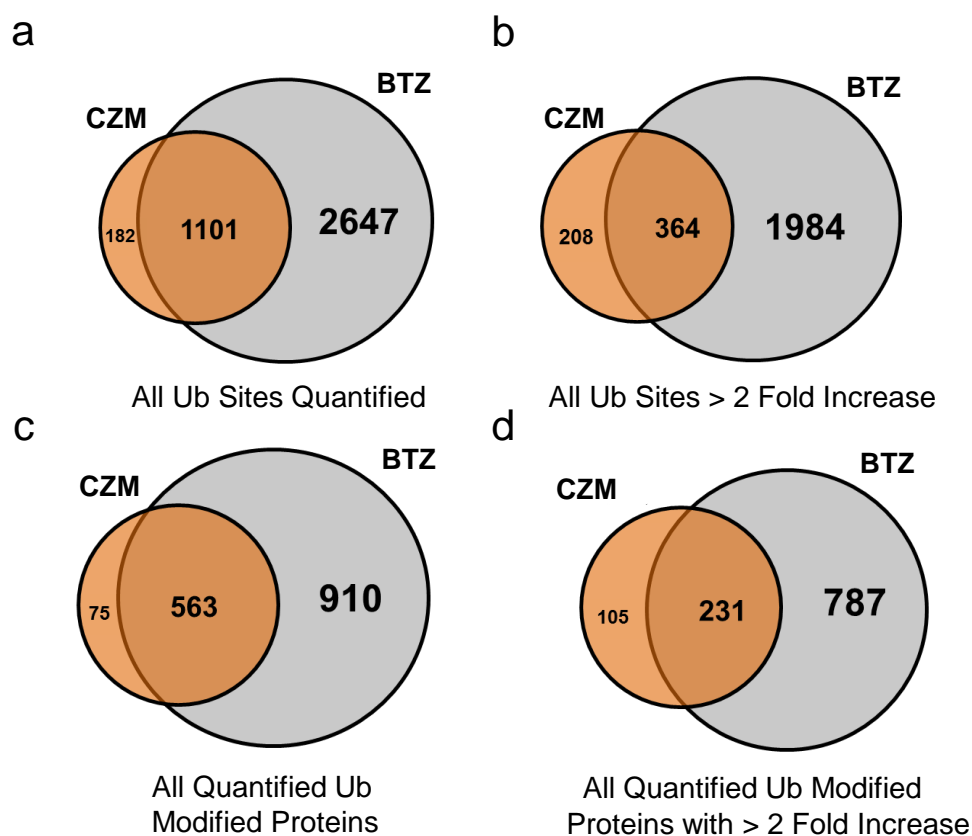
Supplementary Figure 15: Summary of GI₅₀ values from NCI60 screen. The average GI₅₀ (3.3 μM) was set to zero, and the sensitivity of individual cell types relative to the average is plotted on a linear scale.

Supplementary Figure 16



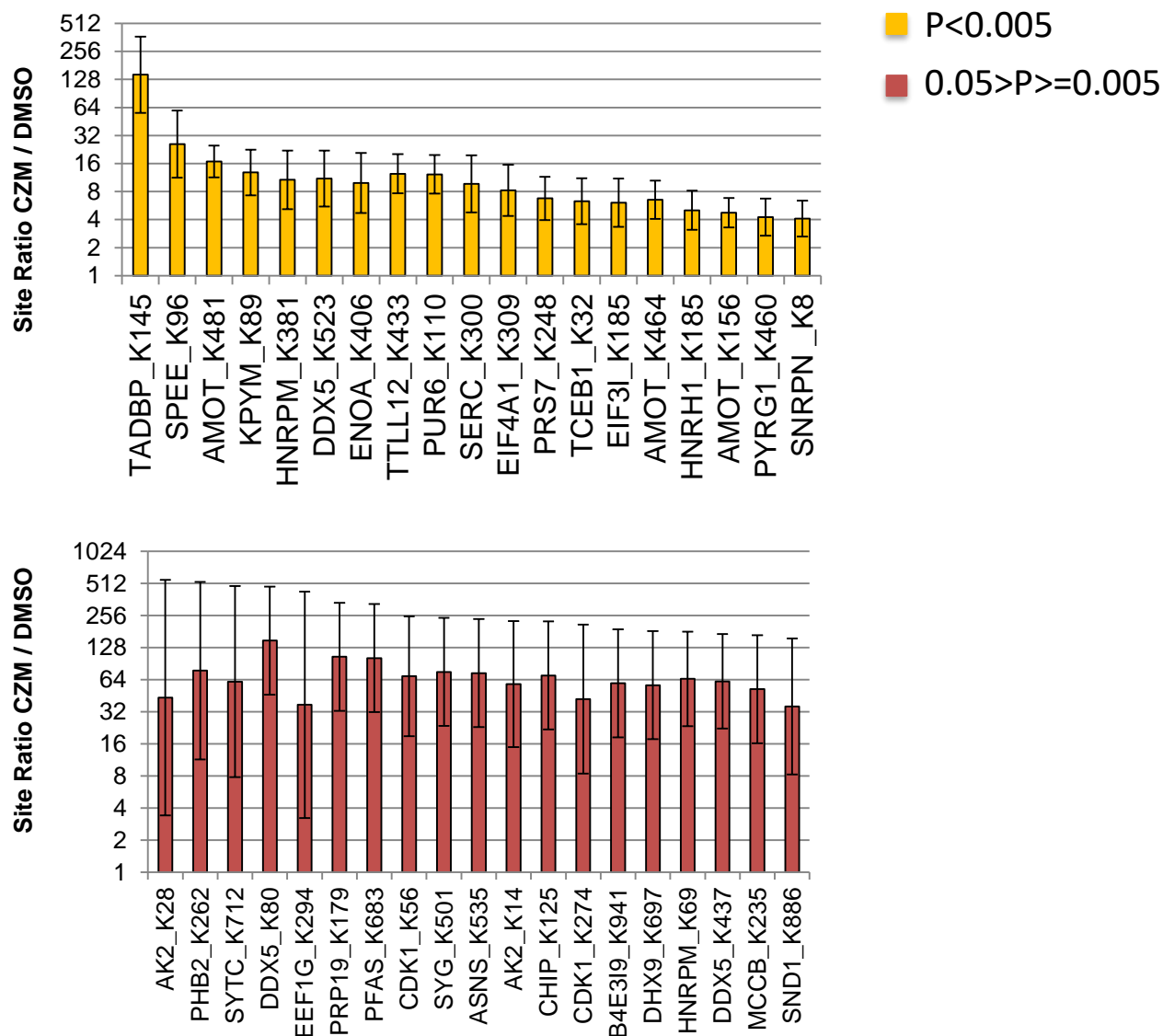
Supplementary Figure 16: Cells lacking Nrf1 are more sensitive to 3021. WT (black) or *nfe2l1*^{-/-} MEF cells (red) were treated with different concentrations of **3021** for 72 hours and then mixed with CellTiter-Glo reagent to quantify viable cells. Measured luminescence values were normalized to DMSO control and data were fitted to a dose-response equation to determine the GI₅₀. Error bars represent s.d., n=3.

Supplementary Figure 17



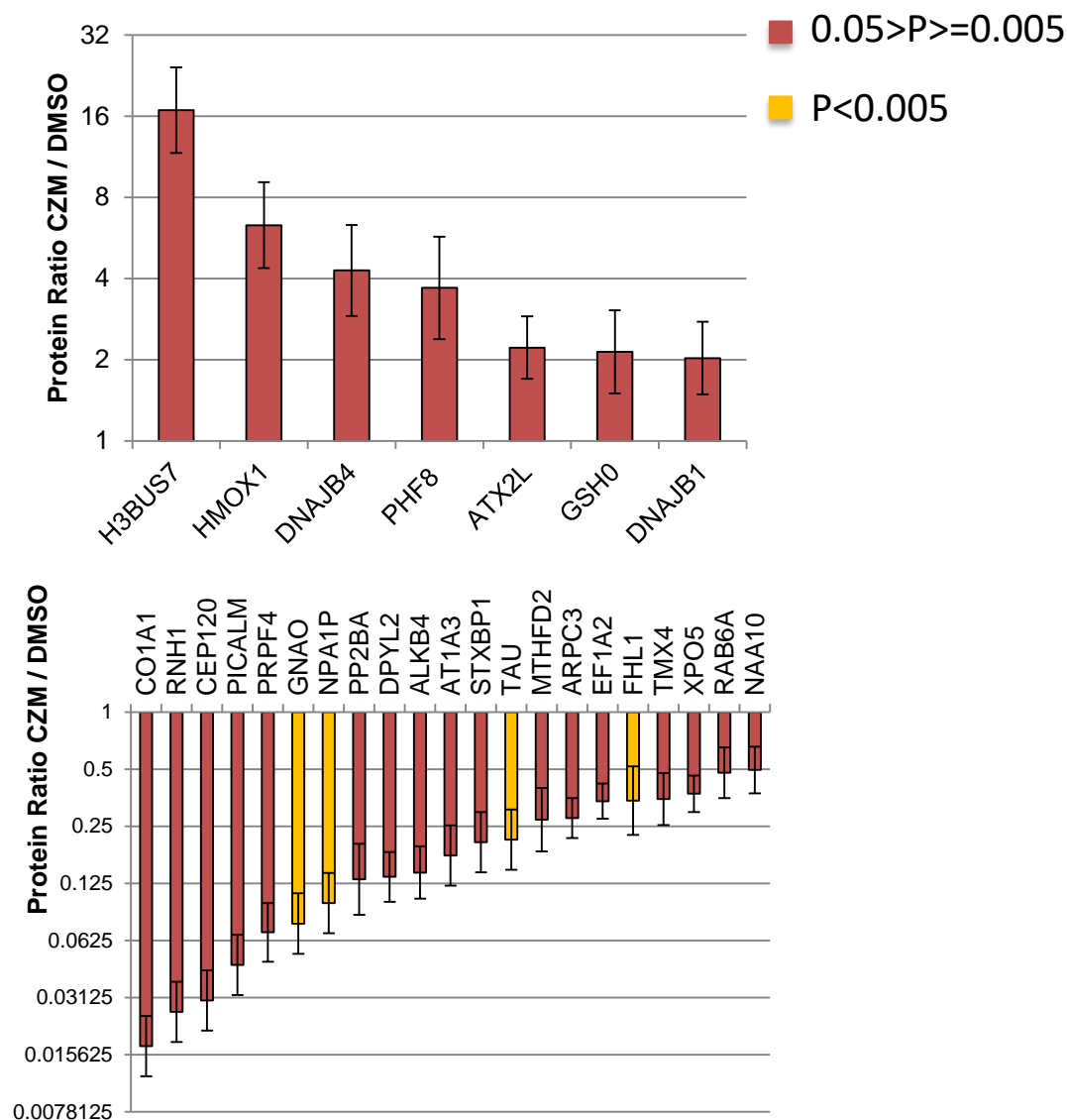
Supplementary Figure 17: Comparison of the effects of CZM and BTZ treatment on ubiquitination site occupancy and protein level. Venn diagram of the data from the proteomic experiment, showing comparison of the effects of capzimin (orange) and bortezomib (gray) on ubiquitination sites (a, b) and protein levels (c, d).

Supplementary Figure 18



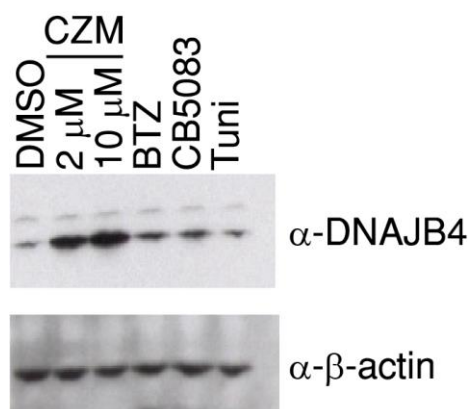
Supplementary Figure 18: Largest changes in ubiquitination site occupancy upon treatment with Capzimin . Bar graphs of the data from the proteomic experiment, showing the ubiquitination sites that showed the largest change in occupancy upon treatment with capzimin. Top panel: sites with p-value ≤ 0.005 . Bottom panel: sites with p-value between 0.05 and 0.005.

Supplementary Figure 19



Supplementary Figure 19: Largest changes in protein levels upon treatment with capzimin. Bar graphs of the data from the proteomic experiment, showing the proteins that showed the largest change in abundance upon treatment with capzimin. Top panel: proteins that increased in level. Bottom panel: proteins that decreased in level. Yellow bars: proteins with p-value ≤ 0.005 . Red bars: proteins with p-value between 0.05 and 0.005.

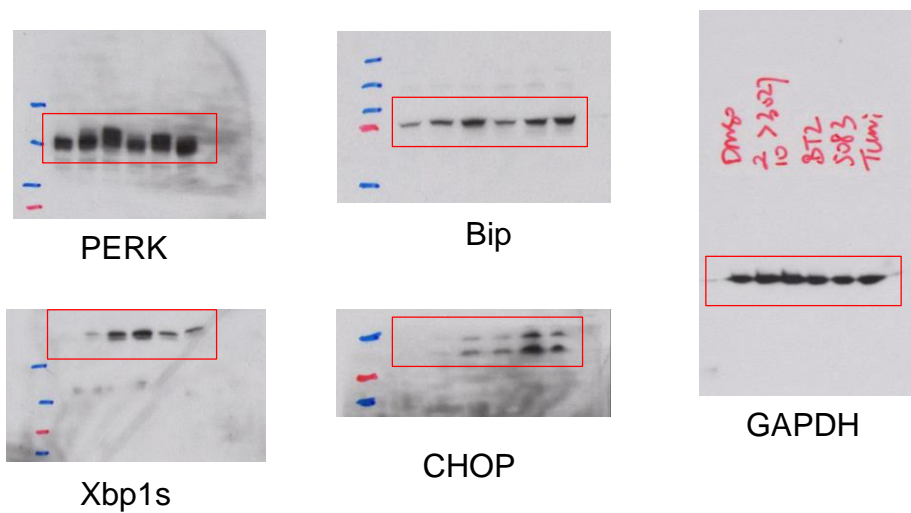
Supplementary Figure 20



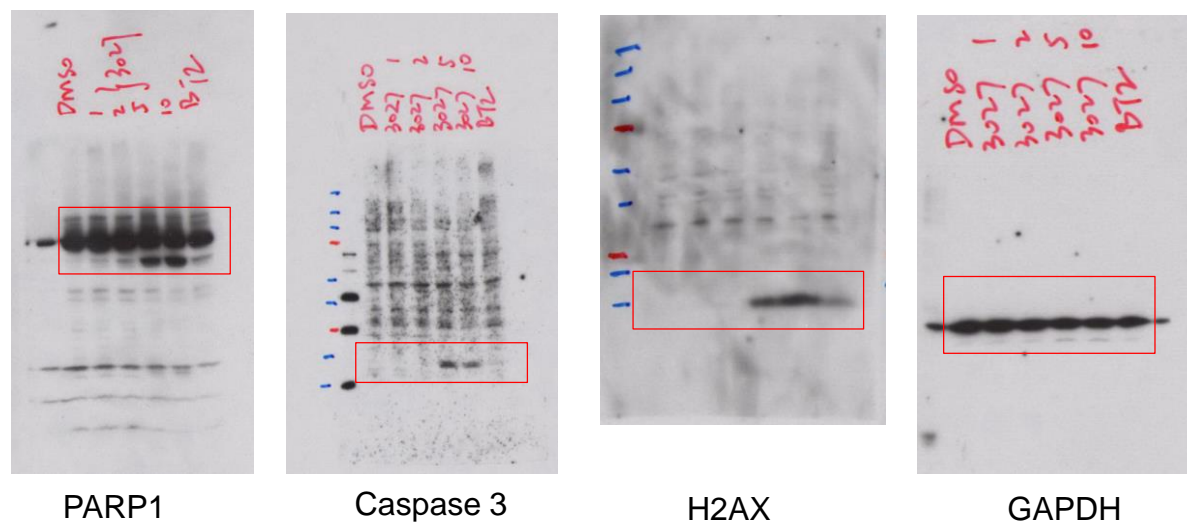
Supplementary Figure 20: Capzimin promoted accumulation of DNAJB4. 293T cells were treated with different concentrations of capzimin (2 or 10 μM), BTZ (1 μM), CB5083 (10 μM), or Tunicamycin (2 μg/ml) for 8 hrs. Western blot analyses were performed using antibodies against DNAJB4 and β-actin.

Supplementary Figure 21

For Fig. 3d



For Fig. 4c



Supplementary Figure 21: Full Western blot images